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In Vitro Growth of Swine Roundworm Larvae, *Ascaris suum:* Cultivation Techniques and Endocrine Regulation

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ABSTRACT: A stationary, multi-well bioassay for growth and development of *Ascaris suum* larvae has proven markedly sensitive for the study of endocrinological and pharmacological substances. Experiments were designed to assess physical factors of the cultivation procedure (larval density and type of flask) as well as the temporal sensitivity of the larvae to potential growth stimulants or inhibitors. Larvae at lower density (100/ml) did not grow as quickly as larvae at higher density (400/ml), coincident with their conversion to anaerobic metabolism. Large stationary flasks, irrespective of cap type, were similar in larval growth patterns. The present study demonstrated that the ecdysteroid agonists, RH-5849, but not RH-5992, had a biphasic effect on larval growth after 24-hr, premolt exposure; low concentrations (5 ng/ml) increased growth, and high concentrations (≥50 ng/ml) decreased growth. Addition of 20-hydroxyecdysone (20-OH) after the third molt also decreased growth of the fourth-stage larvae; ecdysone did not share this effect over the same dose range. Decreased growth, when exposure after molting to 20-OH or an agonist, suggests that alternative pathways might be disrupted by the presence of these compounds.

KEY WORDS: Nematode, *Ascaris*, ecdysteroids, plumbagin, dibenzoyl hydrazines.

In nematodes, ecdysis is defined broadly as the deposition of a new cuticle under the old one, and then the removal of the old cuticle, or exsheathment (Willett, 1980). Regulation of these events in parasitic nematodes is understood incompletely but apparently involves neural (Davey, 1966), hormonal (Dennis, 1977), and enzymatic (Gamble et al., 1989) regulators. The model traditionally used for the study of molting in nematodes is the second molt of *Haemonchus contortus* (Rogers and Head, 1972), but this model involves only the final phase of the ecdysial process, i.e., exsheathment. Hence, synthesis of the new cuticle already is completed while the *Haemonchus* larvae are maintained in a dormant, infective stage, likely obviating any steroidal involvement in the final, short-term (30 min) process of exsheathment. In *Nematospoides dubius* (Dennis, 1977), *Ascaris suum* (Fleming, 1985a), and *Dirofilaria immitis* (Barker et al., 1990), very low concentrations of exogenous ecdysteroids enhanced ecdysis in vitro. Additionally, steroidal regulators also might serve as nonspecific growth modulators independent of molting in nematodes.

An in vitro growth bioassay system was developed (Fleming, 1985b) based on cultivation techniques of third- to fourth-stage *A. suum* larvae (Urban and Douvres, 1981), a developmental transition that encompasses both cuticle deposition as well as exsheathment. With this bioassay, the effects of steroids (Fleming, 1985a, b), biogenic amines (Fleming, 1988), and insect molting inhibitor (Fetterer and Fleming, 1991) have been examined in dose–response experimental designs, with exposure to the drugs limited to a brief (24 hr) premolt period. However, various aspects of the cultivation process have not been examined specifically relative to previously defined hormonal responses. These include aspects of larval culture density, optimal culture vessel, and postmolting or long-term exposure to potential regulators. These regulators comprise ecdysone, 20-hydroxyecdysone (20-OH), the ecdysteroid agonists (dibenzoyl hydrazines) RH-5849 (Wing, 1988) and RH-5992 (Smagghe et al., 1995), and plumbagin (5-hydroxy, 2-methyl-1,4-napthoquinone), an extract of plants of the *Plumbago* spp. that inhibits insect development (Kubo et al., 1983; Joshi and Sehnal, 1989) and demonstrates antileishmanial activity (Croft et al., 1985). The experiments herein were designed to explore these variables on the in vitro growth of *A. suum* larvae in our previously defined bioassay (Fleming, 1985b).

Materials and Methods

General larval cultivation procedures

Seven days after *per os* inoculation of 100,000 decoated and embryonated eggs of *A. suum* (Urban et al., 1981), third-stage larvae were recovered from porcine lungs by Baermannization. These larvae were rinsed

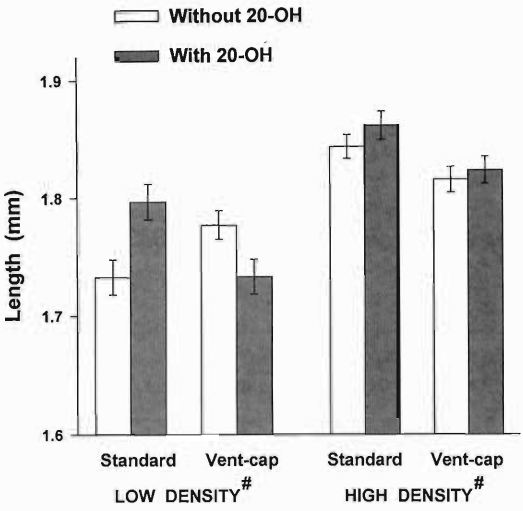


Figure 1. Mean lengths ± SEM of fourth-stage *A. suum* larvae (*N* = 100/group) from 250-ml standard or vent-cap culture flasks at low or high density (100 or 400 larvae/ml) and in the presence (5 ng/ml) or absence of 20-hydroxyecdysone (20-OH). Treatments with the same symbol (#) are significantly different ($P < 0.05$).

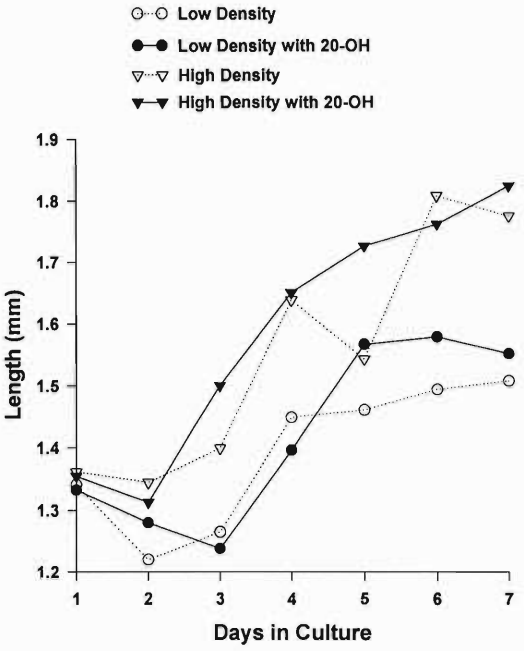


Figure 2. Growth curves of third- to fourth-stage *A. suum* larvae (*N* = 100 day/group) multi-well plates at 2 different densities (100 or 400 larvae/ml) and in the presence (5 ng/ml) or absence of 20-hydroxyecdysone (20-OH) over 7 days of cultivation.

in Dulbecco's phosphate-buffered saline (DPBS) with penicillin/streptomycin/genomycin 5 times (1 hr each rinse) and rinsed 5 times with fresh DPBS (Urban and Douvres, 1981). After sedimentation, medium was aspirated, and larvae were resuspended in RPMI-1640 with 50 μg/ml of cholesterol (Urban et al., 1983) and placed in either 24 multi-well culture plates or 250-ml stationary flasks. Cultures were incubated for 7 or 14 days at 37° C in 5% CO_2:95% air, and then larvae were fixed in hot buffered formalin. Subsamples of larvae (*N* = 25/well or 100/flask) were magnified and projected onto the screen of a computer-linked digitizer (R & M Biometrics, Nashville, Tennessee), and fourth-stage larvae were measured. Data were analyzed by analysis of variance, and means were compared with Duncan's multiple range test (Freund and Little, 1981), with the exception of the time-course experiment. Because this experiment required sequential sampling of the same flasks, the analysis utilized a generalized linear model that recognized repeated measurements (n = 7) from the same experimental unit, i.e., culture flask (Gill and Hafs, 1971). Means were considered significantly different at $P < 0.05$.

Large flask cultivation

Stationary 250-ml flasks, with or without vent caps (Corning Incorp., Corning, New York), were filled with 50 ml of medium and 100 or 400 third-stage larvae/ml. Half of each group of these flasks also were supplemented with 20-OH (5 ng/ml), a concentration that maximally stimulated growth in multi-well cultures (Fleming, 1985b). Cultures were terminated after 7 days of incubation.

Time course

Multi-well plates were incubated with 100 or 400 larvae/well (2 ml/well) with the addition or absence of 5 ng/ml of 20-OH. Eight wells from each treatment group were fixed each day during 7 days of incubation.

Insect molting agonists

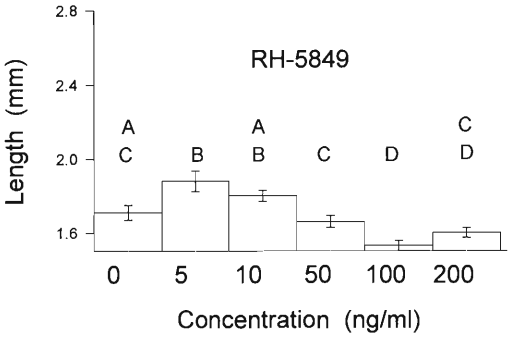
Lung larvae (300/well) were incubated for 24 hr in multi-well plates that were coated with increasing concentrations of the insect molting agonists RH-5849 or RH-5992. Larvae were removed from culture wells and centrifuged, medium was aspirated, and larvae were returned to fresh medium and untreated culture plates for an additional 6 days of cultivation, then fixed and measured.

Postmolting hormonal treatment

Lung larvae were incubated in multi-well plates for 4 days, after which the majority had molted to fourth-stage larvae. Medium and larvae then were transferred to new plates that were coated with various concentrations of ecdysone, 20-OH, or ethanol vehicle and dried. After 3 days of further incubation, larvae were fixed and measured.

Two-week incubation

Third-stage larvae were incubated in multi-well plates for 1 wk and then transferred to new plates that



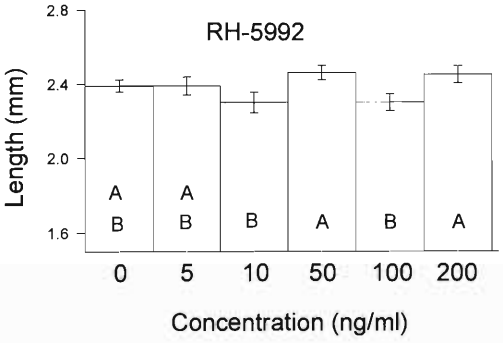
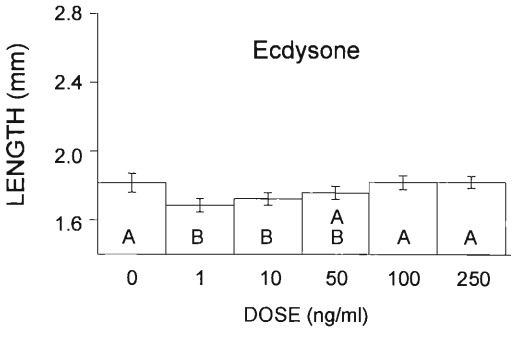


Figure 3. Mean lengths ± SEM of fourth-stage *A. suum* larvae (N = 100/dose) from 7-day multi-well cultures that were exposed to RH-5849 or RH-5992 for the initial 24 hr. Columns with different letters are significantly different ($P < 0.05$).



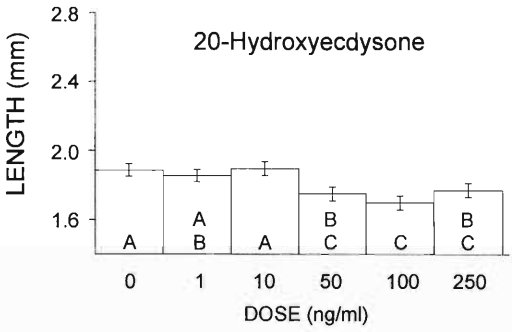


Figure 4. Mean lengths ± SEM of fourth-stage *A. suum* larvae (N = 100/dose) from multi-well plates after 7 days of incubation when ecdysone or 20-hydroxyecdysone (5 ng/ml) was added to culture medium on day 4 of incubation. Columns with different letters are significantly different ($P < 0.05$).

were coated with various concentrations of 20-OH, plumbagin, or ethanol vehicles. After 7 days of further incubation, larvae were fixed.

Results

The type of large cultivation flask or presence of 20-OH had no significant effect on the growth of third- to fourth-stage larvae. However, high cultivation density in large culture flasks resulted in significantly increased larval size (Fig. 1).

Stationary multi-well larval cultures at the higher density, irrespective of the presence of 20-OH, were significantly larger (15%) than cultures at low density with or without 20-OH, after 7 days of cultivation (Fig. 2). Divergence of growth rates occurred primarily after day 2.

Brief exposure to the ecdysteroid agonist RH-5849 generally decreased the subsequent size of larvae, although the sister compound, RH-5992, had no consistent effect on growth over the same dose range (Fig. 3).

Addition of ecdysone after molting (day 4) significantly decreased growth at the lower concentration, but the higher concentrations were similar to control values (Fig. 4). In contrast, a similar temporal addition of 20-OH significantly decreased growth at the higher concentrations (Fig. 4). Growth of larvae from the multi-well plates was comparable to growth of larvae from the culture flasks (Fig. 1).

The exposure of fourth-stage larvae to 20-OH from days 7 through 14 of cultivation resulted in significant decreased growth at only the intermediate concentration of 100 ng/ml (Fig. 5). Plumbagin, with the same regimen of exposure and cultivation, resulted in fourth-stage larvae that were consistently 10% longer than control larvae at all drug concentrations.

Discussion

The enhanced growth of the *A. suum* larvae at the 4-fold higher density suggests that certain metabolic processes function as positive stimuli

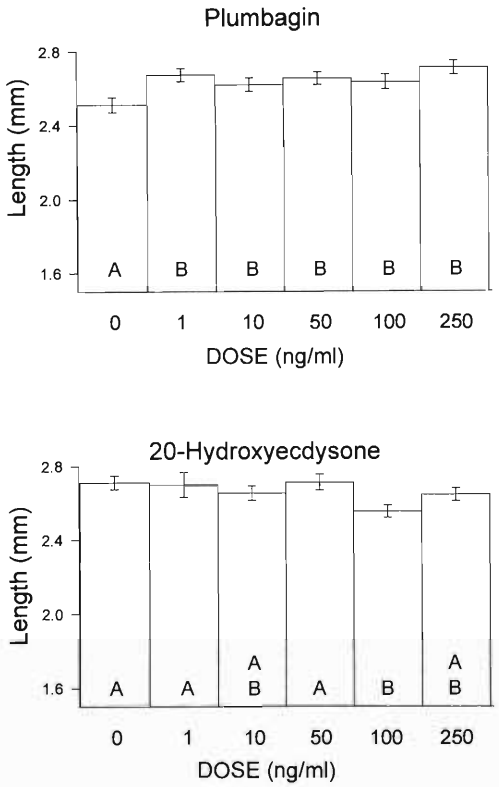


Figure 5. Mean lengths ± SEM of fourth-stage *A. suum* larvae (N = 100) from multi-well plates after 14 days of incubation when plumbagin or 20-hydroxyecdysone was added to medium on day 7. Columns with different letters are significantly different ($P < 0.05$).

for growth. Significantly, this positive response in the time-course experiment is concurrent with the conversion of larval metabolism from aerobic to anaerobic (Komuniecki and Vanover, 1987; Komuniecki and Harris, 1995). Potentially, the higher concentration of larvae more rapidly converts the culture environment to an anaerobic condition, enhancing larval development into the fourth stage. Alternatively, the more rapid conditioning of the media with fermentation products, particularly those associated with the adult parasite (Komuniecki and Vanover, 1987; Vanover-Dettling and Komuniecki, 1989), might also contribute to a more favorable environment at higher larval density.

The results relative to the postmolting exposure to 20-OH support the hypothesis that the precise timing of this specific hormone is critical for its growth enhancement potential. When 20-OH is applied earlier in the cultivation procedure (day 1) and for a short duration (24 hr), larvae molted earlier and grew longer (Fleming, 1985a). The later and longer exposure of 20-OH reported herein, however, did not elicit any growth enhancement. These time- and dose-specific effects argue for an early endogenous role of 20-OH in molting and development in nematodes.

The biphasic response of RH-5849 is similar to that observed with 20-OH in this bioassay (Fleming, 1985b), suggesting an ecdysonergic property in nematodes at low concentrations. The general lack of response with RH-5992 is similar to that reported as differential physiological responses of these 2 ecdysteroid mimics in a variety of insects, including *Plodia interpunctella* (Silhacek et al., 1990), *Spodoptera exigua* (Smagghe and Degheele, 1994), and *Leptinotarsa decemlineata* (Smagghe et al., 1995). Hence, RH-5992 might have distinct ecdysteroid activity that is not present in this stage of *Ascaris*, thus exhibiting a functional site distinguishing these insects from nematodes.

Hormonal, temporal, and cultivation factors have been further explored to define larval growth of *A. suum* and target windows in which specific responses are expressed. Prolonged or postmolting exposure to ecdysteroids inhibited growth in *A. suum* larvae in contrast to its effect with premolting incubation.

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Literature Cited

Barker, G. C., D. J. Chitwood, and H. H. Rees. 1990. Ecdysteroids in helminths and annelids. Invertebrate Reproduction and Development 18:1–11.

Croft, S. L., A. T. Evans, and R. A. Neil. 1985. The activity of plumbagin and other electron carriers against *Leishmania donavavani* and *Leishmania*

mexicana amazoneusis. Annals of Tropical and Medical Parasitology 79:651–653.

Davey, K. G. 1966. Neurosecretion and molting in some parasitic nematodes. American Zoologist 6: 243–249.

Dennis, R. D. 1977. Insect morphogenetic hormones and developmental mechanisms in the nematode, *Nematospiroides dubius.* Comparative Biochemistry and Physiology 52A:53–56.

Fetterer, R. H., and M. W. Fleming. 1991. Effects of plumbagin on development of the parasitic nematodes *Haemonchus contortus* and *Ascaris suum.* Comparative Biochemistry and Physiology 100C:539–542.

Fleming, M. W. 1985a. *Ascaris suum:* role of ecdysteroids in molting. Experimental Parasitology 60: 207–210.

———. 1985b. Steroidal enhancement of growth in parasitic larvae of *Ascaris suum:* validation of a bioassay. Journal of Experimental Zoology 233: 229–233.

———. 1988. Hormonal effects on the in vitro larval growth of the swine intestinal roundworm, *Ascaris suum.* Invertebrate Reproduction and Development 14:153–160.

Freund, R. J., and R. C. Little. 1981. SAS for Linear Models. SAS Institute, Cary, North Carolina. 231 pp.

Gamble, H. R., J. P. Purcell, and R. H. Fetterer. 1989. Purification of a 44 kilodalton protease which mediates the ecdysis of infective *Haemonchus contortus* larvae. Molecular and Biochemical Parasitology 33:49–58.

Gill, J. L., and H. D. Hafs. 1971. Analysis of repeated measurements of animals. Journal of Animal Science 33:331–336.

Joshi, N. K., and F. Sehnal. 1989. Inhibition of ecdysteroid production by plumbagin in *Dydercus cingulatus.* Journal of Insect Physiology 35:737–741.

Komuniecki, P. R., and L. Vanover. 1987. Biochemical changes during the aerobic–anaerobic transition of *Ascaris suum* larvae. Molecular and Biochemical Parasitology 22:241–248.

Komuniecki, R., and B. G. Harris. 1995. Carbohydrate and energy metabolism in helminths. Pages 46–66 *in* J. J. Marr and M. Müller, eds. Biochemistry and Molecular Biology of Parasites. Academic Press, London.

Kubo, I., M. Uchicita, and J. L. Klocker. 1983. An ecdysis inhibitor from *Plumbago capensis* (Plumbagnacae): a naturally occurring chitin synthease inhibitor. Agricultural and Biological Chemistry 47:911–913.

Rogers, W. P., and R. Head. 1972. The effect of the stimulus for infection on hormones in *Haemonchus contortus.* Comparative and General Pharmacology 3:6–10.

Silhacek, D. L., H. Oberlander, and P. Porcheron. 1990. Action of RH-5849, a nonsteroidal ecdysteroid mimic, on *Plodia interpunctella* (Hübner) in vivo and in vitro. Archives of Insect Biochemistry and Physiology 15:201–212.

Smagghe, G., G.-A. Bohm, K. Richter, and D. Degheele. 1995. Effect of nonsteroidal ecdysteroid agonists on ecdysteroid titer in *Spodoptera exigua* and *Leptinotarsa decemlineata.* Journal of Insect Physiology 41:971–974.

———, **and D. Degheele.** 1994. Effects of the ecdysteroid agonists RH-5849 and RH-5992, alone and in combination with a juvenile hormone analogue, pyriproxyfen, on larvae of *Spodoptera exigua.* Entomologia Experimentalis et Applicata 72:115–123.

Urban, J. F., Jr., and F. W. Douvres. 1981. In vitro development of *Ascaris suum* from third- to fourth-stage larvae and detection of metabolic antigens in multi-well culture systems. Journal of Parasitology 67:800–806.

———, ———, **and X. Shoutai.** 1983. Analysis of culture requirements of *Ascaris suum* larvae using stationary multi-well system: increased survival, development and growth with cholesterol. Veterinary Parasitology 14:33–42.

———, ———, **and F. G. Tromba.** 1981. A rapid method for hatching *Ascaris suum* eggs in vitro. Proceeding of the Helminthological Society of Washington 48:241–243.

Vanover-Dettling, L., and P. R. Komuniecki. 1989. Effect of gas phase on carbohydrate metabolism of *Ascaris suum* larvae. Molecular and Biochemical Parasitology 36:29–40.

Willett, J. D. 1980. Control mechanisms in nematodes. Pages 197–225 *in* B. M. Zuckermman, ed. Nematodes as Biological Models. Vol. I. Academic Press, New York.

Wing, K. D. 1988. RH 5849, a nonsteroidal ecdysone agonist: effects on a *Drosophila* cell line. Science 241:467–469.